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## **INTRODUCTION**

Sik is an intracellular tyrosine kinase that was first identified in a screen for tyrosine kinases in intestinal epithelial cells (25). Although it is related to the Src family and contains SH2 and SH3 domains, it has a very short unique amino terminus and is not myristoylated (30). Sik expression is restricted to differentiating epithelial cells and it is found in the skin and all linings of the alimentary canal. Addition of calcium to cultured primary mouse keratinocytes induces cell differentiation and rapid activation of Sik (31). Overexpression of Sik in an embryonic mouse keratinocyte cell line resulted in increased expression of the differentiation marker filaggrin during calcium-induced differentiation, suggesting that Sik is involved in a signal transduction pathway that may promote differentiation (31).

The human orthologue of Sik is also called BRK (breast tumor kinase) (18, 19). Like Sik, BRK is expressed in normal epithelial cells that are undergoing terminal differentiation. BRK expression has been detected in colon tumors (18), breast tumors (2) and melanomas (8, 16). While BRK appears to play a role in signal transduction in normal epithelial linings, its overexpression appears to be linked to the development of a variety of epithelial tumors. The seemingly paradoxical roles of Sik/BRK during differentiation and tumorigenesis are poorly understood.

During the last funding period, we determined that Sik is a nuclear tyrosine kinase that phosphorylates Sam68. Sam68 (Src-associated in mitosis, 68 kDa) is an RNA binding protein that was identified as a major target of Src during mitosis when the nuclear membrane breaks down (10, 29). Sam68 contains a STAR (Signal Transduction and Activation of RNA) domain of approximately 200 amino acids that is also referred to as the GSG (GRP33/Sam68/GLD1) domain (reviewed in 33). A conserved KH (hnRNPK Homology) RNA binding domain is located within the STAR domain. KH domains play crucial roles in proteins encoded by several developmentally important genes, including the human FMR1 (fragile X mental retardation syndrome) gene (7), the mouse Qk1 (quaking) gene required for myelination (9), the *C. elegans* GLD-1 gene that is required for germ cell differentiation (13), and the *Drosophila* Who/How gene required for muscle differentiation (1). The Sam68 KH domain is important for its nuclear localization and self-association (5). Sam68 also contains several proline rich sequences that facilitate interactions with SH3 and WW domain containing proteins, and multiple tyrosines in its carboxy terminus (23).

Sam68 can bind and be phosphorylated by Src and Src family members (23) and the Tec family member Itk (3) following nuclear breakdown during mitosis. It has also been found to be phosphorylated on serine and threonine residues by Cdc2/cyclin B complexes (22). Sam68 preferentially binds RNA with UAAA motifs (17, 22), but tyrosine phosphorylation by Fyn impairs its RNA binding ability (34). Sam68 also associates with phospholipase C $\gamma$ , and the adaptor proteins Grb2 (23) and Nck (15). Sam68 appears to play a role posttranscriptional control of gene expression, as it was recently shown to be a functional homologue of the HIV-1 Rev protein that is involved in nuclear export of RNA containing the Rev response element (21).

Sik/BRK is the only identified tyrosine kinase that colocalizes with Sam68 within the nucleus. Below we show that Sik/BRK is active within the nucleus and that it can phosphorylate Sam68 *in vivo*. Phosphorylation of Sam68 within the nucleus may have important physiological significance and may contribute to the posttranscriptional control of gene expression in breast cancers.

## **BODY**

### **Materials and Methods**

#### **Expression Constructs**

For the preparation of the mutant sik cDNAs, we used the oligonucleotide-mediated Altered sites in vitro mutagenesis system (Promega). The sik cDNA was cloned into the pAlter plasmid, and the oligo, 5'-CACCAGGTTTGAGAACC-3', with a substitution of A for T resulting in substitution of the tyrosine at position 447 to phenylalanine, was used to generate the Sik Y-F construct. This type of mutation has been shown to lead to constitutive activation of the SRC family of tyrosine kinases (6). Preparation of the kinase defective Sik expression construct Sik K-M is described in (31). Wildtype Sik, Sik Y-F and Sik K-M coding sequences were cloned into the vector pcDNA3. The GST-Sik constructs are described in (31).

#### **Cells and Antibodies**

Primary keratinocytes were isolated from newborn Sencar mice using a trypsin flotation procedure (36) and maintained as described previously (31). NMuMG cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Grand Island, NY) with 4.5 g/L glucose and 10 mcg/ml insulin, 10% fetal bovine serum. NMuMG cells were generally transfected using the LipofectAMINE Reagent (Gibco/BRL). Stable clones of NMuMG cells that were fractionated in Figure 1 were infected with wildtype Sik in the pLXSN retroviral expression vector and selected with G418 (31). HeLa cells were maintained in DMEM with 1.0 mM sodium pyruvate, and 10% bovine calf serum (Hyclone, Logan Utah) and transfected with the vaccinia virus T7 expression system and lysed as previously described (23).

Anti-Sik polyclonal antibodies N-20 and C-17 were obtained from Santa Cruz Biotechnology. Immunoblot analyses were performed with a combination of the two Santa Cruz Biotechnology antibodies, for increased sensitivity. The BRK polyclonal antibody was also obtained from Santa Cruz.

#### **Sik-GST Fusion Protein in Vitro Binding Assays**

GST-Sik fusion proteins were prepared as described previously (31). Cell lysates were precleared by incubating with GST-saturated glutathione beads for 30 min. Precipitations were performed by incubating lysates with GST, GST-SikSH2/3, Sik SH2, or SikSH3 45 min at 4°C followed by incubation with glutathione-Sepharose beads (Amersham) for 30 min. Precipitates were eluted with sample buffer and subjected to SDS-PAGE gel and blotted to Immobilon-P membranes. Sam68 bound to GST-Sik was detected by immunoblotting with anti-Sam68 antibody.

#### **Immunoprecipitations and Immunoblotting**

Cells were lysed in cold immunoprecipitation buffer (20mM Hepes pH 7.4, 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 0.2 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A) for 20-30 minutes on ice with constant agitation. Anti-BRK antibodies (Santa Cruz Biotechnology) and 50 µl of protein G sepharose (Amersham or Pharmacia Biotech) were incubated with 1-2 mg cell lysate 3-16 hours at 4°C. As controls, lysates were incubated with sepharose beads and rabbit serum, rabbit IgG, or alone. Immune complexes were washed 3 times with immunoprecipitation buffer (containing 0.1% Triton X-100) and recovered by boiling 3 minutes in 2x gel sample buffer (Tris pH 6.8, SDS, glycerol, bromophenol blue, water) and 10% 2-mercaptoethanol. Proteins were analyzed by

SDS-PAGE and transferred to polyvinylidene-difluoride (Immobilon, Millipore, Bedford, MA) filters. The filters were blocked 1-2 hours in 5% nonfat dry milk in buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.1% Tween 20) at room temperature and incubated with anti-Sam68 AD1 antibodies, followed by HRP-conjugated donkey anti-rabbit antibodies (Amersham). The reaction was visualized with SuperSignal ULTRA chemiluminescence substrate (Pierce).

### **Immunofluorescence**

Cells were grown on chamber slides (Falcon) and fixed in methanol at -20°C for 5 minutes or Carnoy's fixative for 5 minutes at room temperature. Cells transfected with GFP constructs were fixed in 4% paraformaldehyde for 5 minutes at room temperature and permeabilized in 50% methanol/50% acetone for 15 minutes at -20°C. Slides were blocked in 2% goat serum or 3% BSA in TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 30-40 minutes. Slides were then incubated with anti-BRK or anti-Sam68 antibodies (1:250) overnight at 4°C, washed, incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories) (1:250) for 1 hour at room temperature, washed, blocked 30 minutes with blocking reagent (from DuPont NEN), and incubated with Streptavidin-HRP (DuPont NEN) (1:100). After washing, tyramide amplification was performed using the TSA-Indirect Kit (DuPont NEN) according to manufacturer's directions. Reactions were visualized with rhodamine-avidin (Vector Laboratories) (1:500) and slides were mounted with Vectashield mounting medium (Vector Laboratories). Controls were performed by incubation of cells with rabbit serum, rabbit IgG, or in blocking buffer alone.

For co-staining, cells were stained as above with anti-BRK and visualized with rhodamine, followed by incubation with anti-Sam68 (Transduction Laboratories) (1:50) for 1 hour at room temperature and anti-mouse IgG FITC conjugate (Sigma) (1:64) and analyzed by confocal microscopy.

Cells transfected with GFP-Sam68 and Sik constructs were stained overnight with anti-phosphotyrosine antibody conjugated to horseradish peroxidase (RC20-HRPO, Transduction Laboratories) (1:2000) at 4°C, incubated with biotinyl tyramide from TSA-Indirect Kit, and visualized with rhodamine-avidin (1:500). Slides were mounted or stained with anti-Sik antibodies (Santa Cruz) (1:250) amplified using TSA-indirect kit (DuPont NEN) as before and visualized with streptavidin Alexa 350 conjugate (Molecular Probes) (1:500). Nuclei were stained with DAPI (Boehringer Mannheim) for 3 minutes and washed before mounting.

### **Subcellular Fractionation**

Cells were washed 2 times in 1x PBS and 1 time in hypotonic lysis buffer (HLB: 20 mM Tris-HCl pH 7.5, 1 mM MnCl<sub>2</sub>, 2 mM EGTA) for 5 minutes on ice. Cells were then treated with 1.5 ml of HLB (with 20 mcg/ml leupeptin, 1 mM PMSF) and shaken for 20 minutes on ice. Cells were scraped and homogenized in a Dounce homogenizer (50-60 strokes) and spun for 10 minutes at 2300 rpm, 4°C. The supernatant was centrifuged in Beckman ultracentrifuge at 28000 rpm, 4°C for 45 minutes. The pellet was washed in 1 ml of HLB, spun 4 minutes at 5000 rpm at 4°C, and resuspended in 1 ml Dignum buffer (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MnCl<sub>2</sub>, 0.1 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, 1 mM NaVO<sub>4</sub>). After shaking for 15 minutes at 4°C, samples were spun at 14000 rpm for 10 minutes at 4°C. The supernatant was kept as nuclear fraction. The supernatant from ultracentrifuge was kept as cytosolic fraction. The pellet was dissolved in 1 mL cell lysis buffer (CLB) with 1% SDS and 0.1%

sodium deoxycholate (CLB: 1 % Triton X-100, 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 1mM DTT, 2 mM sodium vanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 mcg/ml leupeptin, 1 mM PMSF, 10 mcg/ml aprotinin) for 15 minutes at 4°C with constant agitation. After centrifugation at 14000 rpm the supernatant was kept as particulate fraction.

## **Results**

### **BRK associates with Sam68 in human tumor cell lines**

BRK is expressed breast and colon tumors and tumor cell lines. When we performed immunofluorescence studies, we determined that BRK was localized to distinct nuclear structures in the MCF-7 breast cancer cell line and the HT29 colon adenocarcinoma cell line (Figure 1A). The RNA binding protein Sam68 was previously observed in novel nuclear structures that looked similar. The structures were termed SNBs (Sam68 Nuclear Bodies), and they are distinct from coiled bodies, gems and PML nuclear bodies (4). Using confocal microscopy we determined that BRK colocalizes with Sam68 in SNBs found in the HT29 colon adenocarcinoma cell line (Figure 1B).

To determine if endogenous Brk also associates with Sam68 in breast and colon tumor cell lines, we performed coimmunoprecipitation experiments using total cell lysates from the human colon carcinoma cell line HT29 and the breast tumor cell line MCF7. A band corresponding to Sam68 was detected following immunoprecipitation with anti-BRK antibody, but with not with normal rabbit serum or when 1° antibody was omitted (Fig. 2A). We detected a significant increase in the amount of Sam68 that coprecipitated with BRK when nuclear protein fractions were used (Figure 2B).

### **Sik is a nuclear tyrosine kinase**

Sik expression has been detected in skin and all linings of the alimentary canal. Primary cultured newborn mouse keratinocytes express endogenous Sik protein, while Sik is not expressed in the normal murine mammary gland cell line (NMuMG) (12). To examine the intracellular localization of Sik, primary keratinocytes or stable clones of NMuMG cells transfected with wildtype Sik, were fractionated into nuclear, cytosolic (S100) and particulate (P100) fractions. Immunoblotting was performed with proteins from each fraction and total cell lysates and antibodies recognizing the membrane protein Met, the cytosolic protein cytoplasmic phospholipase A2 (cPLA2), the nuclear protein Sam68, and Sik (Figure 3). Met, cPLA2, and Sam68 antibodies reacted with their target proteins in the expected fractions. In primary keratinocytes, the endogenous Sik protein was concentrated in the nuclear fraction. In selected pools of transfected NMuMG cells, Sik protein was detected throughout the cell, in particulate, cytosolic, and nuclear fractions.

### **Sik phosphorylates Sam68 in vivo**

We further examined Sik localization and activity in the NMuMG cell line, which does not express Sik. Cells were transiently transfected with wildtype, putative activated (Y-F), and kinase defective (K-M) Sik. The activated form of Sik contains a Tyr to Phe substitution of the putative regulatory tyrosine at position 447 of Sik, while the kinase defective Sik contains a substitution of a conserved Lys at position 219 to Met (31). The different Sik expression constructs were co-transfected with a green fluorescent protein (GFP) – Sam68



fusion construct (4), and tyrosine phosphorylation was examined using immunoblotting. Tyrosine phosphorylated GFP-Sam68 was detected in total cell lysates from NMuMG cells cotransfected with wildtype Sik or Sik Y-F and the GFP-Sam68 expression construct, but not in cells cotransfected with vector alone or the kinase defective Sik K-M construct (Figure 4 A). Although transfection efficiencies were equivalent for the different Sik constructs, the Sik K-M construct is reproducibly expressed at lower levels than the other Sik constructs, suggesting that it may produce a less stable protein in NMuMG cells. Sik was found to coimmunoprecipitate with Sam68, confirming its association (Figure 4B).

Using confocal microscopy we examined the localization and tyrosine phosphorylation of Sam68 in the presence of wildtype Sik, Sik Y-F, and Sik K-M (Figure 5, part B). Co-transfection of the GFP expression vector and the empty Sik expression vector resulted in diffuse GFP throughout the cell (Fig. 5M) and no detectable anti-phosphotyrosine staining (Fig. 5N). In contrast, the GFP-Sam68 fusion protein was localized to the nucleus (Fig. 5A, E, I). Phosphotyrosine was readily detected in the nuclei of cells transfected with wildtype and activated forms of Sik (Fig. 5B, F), but not kinase defective Sik K-M construct (Fig. 5J). Sik phosphorylated proteins colocalized with Sam68 (Fig 5C, G). The pattern of wildtype Sik expression is shown in panel C of Figure 5A, where Sik is visualized by avidin-Alexa 350 (blue). Transfected wildtype Sik is present in the nucleus and at the membrane, consistent with the fractionation studies shown in Figure 3.

#### **Sik-Sam68 Interaction is mediated by Both the SH3 and SH2 domains**

To determine which part of Sik interacts with Sam68, different domains of Sik were expressed in bacteria as GST fusion proteins. Fusion proteins containing the Sik SH3 domain, the SH2 domain, and both the SH3 and SH2 domain were prepared. Lysates from NMuMG cells transfected with wildtype Sik, Sik Y-F, or Sik K-M and GFP-Sam68 expression constructs were incubated with the GST fusion proteins and then with glutathione-sepharose. The phosphotyrosine containing proteins associated with the GST-Sik fusions were detected by anti-phosphotyrosine antibody immunoblotting (Figure 6). GFP-Sam68 and the endogenous Sam68 were found to strongly bind the Sik SH3 and Sik SH3/SH2 domain fusion proteins. When Sam68 was coexpressed with wildtype Sik or Sik Y-F, it also bound the SH2 domain of Sik (Figure 6). Thus Sam68 can also associate with the Sik SH2 domain following its phosphorylation by Sik.

#### **PROGRESS IN RELATION TO THE STATEMENT OF WORK/ FUTURE PLANS**

*Task 6: Year 2-3: To determine the biological activity of the brk(sik) kinase in cell lines. We will determine if brk(sik) expression results in altered growth characteristics, cellular transformation or induction of differentiation.*

*Task 7: Years 3-4: To generate MMTV- sik (mutant and wildtype) kinase transgenic mice.*

*Task 8: Year 4: To determine if directing the expression of inappropriate levels or patterns of sik in transgenic mice will result in altered breast development and/or increased tumorigenesis.*

## **Task 6**

The data presented above represent an outgrowth of Task 6 in the original statement of work. We found the SNBs when examining the MCF7 tumor cell line, leading to new questions that were not anticipated at the time the grant was submitted. Sam68 is the first substrate identified for Sik/BRK and its identification should provide important insight about the function of this tyrosine kinase. In the coming year, we would like to pursue these new findings and address the function of Sam68 phosphorylation by Sik/BRK in breast tumor cells.

## **Tasks 7 and 8**

### **Generation of Sik Transgenic Mice**

Although we originally proposed to use the MMTV promoter to generate transgenic mice, we decided to use the keratin 14 promoter to drive expression of wildtype Sik in transgenic mice. Keratin 14 has been reported to be expressed in the mammary gland (14, 28, 35). In carrying out work in tasks 1 and 2, we did not detect Sik in the normal mammary gland. K14 is also expressed in the basal cells of the skin prior to the induction of Sik as cells terminally differentiate (30, 32). By using the K14 promoter we can compare the effects of ectopic expression of Sik in two tissues at the same time, skin and breast. The hypothesis that we are testing is that ectopic expression of Sik may induce abnormal differentiation, changes in cell turnover, and tumor formation.

The transgenic expression construct consists of the full-length Sik cDNA inserted into the BamHI site of pK14-HGH (a gift of Linda Degenstein and Elaine Fuchs) (Figure 7A). The resulting construct is a 6.7 kB EcoRI-HindIII fragment within the cloning site of the pGem 3Z plasmid vector (Promega), which contains approximately 2100 bp of the cytokeratin 14 promoter/enhancer region, followed by approximately 2100 bp of Sik cDNA sequence, followed by 2150 of HGH genomic sequence including three introns and a polyadenylation sequence, followed by 490 bp of cytokeratin 14 3' noncoding region and polyadenylation site. In linearized and purified form, this vector was injected into mouse embryos (11) by Roberta Franks, who runs the UIC Cancer Center mouse core facility. Eighty-six offspring produced and were screened by Southern blot hybridization of tail biopsy DNA samples. Eleven founder transgenic animals were identified. Of these founders, four lines were chosen for further study, corresponding to numbers 20, 60, 70 and 77 in Figure 7 B.

As of yet, no significant morphological changes or changes in proliferation, differentiation or tumorigenesis have been observed in the skin and breast tissue in the Sik wildtype transgenic mice and their offspring. These results parallel results obtained in cell culture. It is possible that stable overexpression of Sik in cells that do not express the appropriate substrates/adaptor proteins. We will further monitor these animals for the development of tumors and examine expression of specific differentiation markers in the breast tissue. In addition, we will examine the status of Sam68 in these animals.

## **CONCLUSIONS**

Nuclear tyrosine kinases have the unique ability to coordinate signal transduction events in the cytoplasm with specific changes in the nucleus. Only a small number of nuclear tyrosine kinases have been identified, including Abl, Rak, Fes, Fer, and Wee1 (reviewed in (20)). The Sik/BRK kinase lacks a clear nuclear localization and has no DNA binding domain (30). Nevertheless, Sik/BRK is present in the nucleus where it associates with and can phosphorylate the RNA binding protein Sam68.

Both Sik and its human orthologue BRK are present in the nuclei of mouse and human cells respectively. NMuMG cells were isolated from the mammary glands of Namru mice and have epithelial growth characteristics and do not form malignant lesions when introduced into nude mice (12). Sik localization is diffuse within the nuclei of immortalized NMuMG cells, while BRK protein appears in specific structures in the HT-29 colon adenocarcinoma cell line (Figure 1 and Figure 4). These data complement earlier studies by Chen and Richard (4), who found that SNBs (Sam68 nuclear bodies) were predominant in transformed cells. SNBs are novel unique dynamic structures that disassemble when transcription is inhibited with actinomycin D (4). When GFP-Sam68 and wildtype Sik are introduced into HT-29 cells, they localize to the SNBs, which become tyrosine phosphorylated (Figure 8C). The consequences of Sam68 tyrosine phosphorylation by Sik within the nucleus and in SNBs need to be explored.

We have shown that Sik can bind Sam68 through both its SH3 and SH2 domains. The binding affinities of specific SH2 domains are influenced by sequence context. For example, Src family members prefer the sequence pTyr-Glu-Glu-Ile, while the SH2 domains of p85 and PLC- $\gamma$  select the general motif pTyr-hydrophobic-X-hydrophobic (26, 27). Using a technique employing degenerate phosphopeptide libraries to predict the specificity of individual SH2 domains, it was determined that the Sik SH2 domain may bind to phosphorylated proteins with p-YEEY, YEDY, YDEY and YDDY motifs (Z. Songyang and L. C. Cantley, personal communication). Interestingly, Sam68 contains the sequence YEDY in its carboxy terminus and this is a putative binding site for the Sik SH2 domain. This sequence may also be the target of Sik, as we show here that Sam68 lacking the carboxy terminus is not phosphorylated by Sik.

RNA binding proteins may regulate gene expression by a number of mechanisms (reviewed in 24). They may alter RNA structure to regulate interaction with trans-acting factors, or provide localization or targeting signals. Although its cellular function is unknown, Sam68 has been shown to be able to functionally substitute for the HIV-1 Rev protein, which plays an essential role in the nuclear export of unspliced and partially spliced viral transcripts and export of the HIV genome (21). This implicates Sam68 in the posttranscriptional regulation of gene expression.

Sam68 is the first substrate identified for the Sik/BRK kinase. Sik/BRK expression is initiated in differentiating cells of the skin and gastrointestinal tract (30), and its activity is induced in primary keratinocytes that are stimulated to differentiate (31). It is possible that Sik phosphorylation of Sam68 within the nucleus regulates an aspect of RNA transport and posttranscriptional gene regulation associated with specific events occurring during normal epithelial cell differentiation. Overexpression of Sik/BRK in breast cancer may result in altered tyrosine kinase activity and phosphorylation of Sam68. It was be important to determine the functional role of Sam 68 tyrosine phosphorylation in future studies.

## Figure Legends

**Figure 1. BRK and Sam68 localize in nuclear structures in breast and colon tumor cell lines.** A. Both BRK and Sam68 are found in nuclear structures in human tumor cell lines. Immunofluorescence was used to examine the localization of endogenous BRK and Sam68 in the MCF-7 (A, B) and HT29 (C, D) cell lines. Cells were fixed and stained with antibodies against BRK (A,C) or Sam68 (B, D). B. BRK localizes to Sam68 nuclear bodies in HT29 cells. Cells were fixed and stained with antibodies against BRK followed by staining with antibodies against Sam68 and analyzed by confocal microscopy. BRK was visualized with rhodamine (A) and Sam68 staining was visualized with FITC (B). A composite of red and green colors (C) shows co-localization of BRK and Sam68 as yellow spots in the nuclei. Nuclei were stained with DAPI (D).

**Figure 2. BRK and Sam68 associate within the nuclei of HT-29 and MCF7 cells.**

A. Sam68 co-immunoprecipitates with BRK in mammary and colon carcinoma cells. Cells were lysed and 1 mg of total cell lysate was incubated with anti-BRK antibodies, normal rabbit serum, or sepharose beads alone as a control for nonspecific binding to beads. The immunoprecipitate was resolved by SDS-PAGE followed by immunoblotting with anti-Sam68 AD1 polyclonal antibodies. B. Nuclear (N) and cytosolic/membrane (C/M) fractions from MCF-7 and HT29 cells were immunoprecipitated with anti-BRK antibodies followed by immunoblotting with anti-Sam68 antibodies. Short exposure (10 seconds) shows interaction in nuclear fraction only. Long exposure (45 seconds) shows weaker interaction in cytosolic/membrane fraction.

**Figure 3. Sik is in the nucleus of nontransformed mouse cells.** Primary keratinocytes and cells of the normal murine mammary gland (NMuMG) cell line that express transfected wildtype Sik were separated into nuclear, cytosolic and particulate (membrane) fractions. Proteins were extracted by boiling in 1x SDS loading buffer, and analyzed by immunoblotting using anti-MET, -cPLA<sub>2</sub>, -Sam68 and -Sik antibodies. In primary keratinocytes, the majority of Sik protein appeared in the nuclear fraction. In stable pools of NMuMG cells expressing transfected Sik, Sik protein was found throughout the cell.

**Figure 4. Sam68 is a substrate for Sik in vivo.**

A. Localization of GFP-Sam68 and wildtype Sik in transfected NMuMG cells. Wildtype transfected Sik antibody binding is visualized with avidin-Alexa 350 (blue) and is present in the nucleus and at the membrane (A; panel C) B. Wildtype Sik and Sik Y-F phosphorylate nuclear proteins colocalizing with Sam68 in NMuMG cells. NMuMG cells were cotransfected with GFP-Sam68 and wildtype Sik, Sik Y-F or kinase defective Sik K-M. Total cell lysates were divided equally and immunoblotted with antibodies against Sam-68, phosphotyrosine and Sik. Tyrosine phosphorylated Sam68 was detected only in lysates containing wildtype Sik or Sik Y-F. B. Sik associates with Sam68. Immunoprecipitations were performed with Sam68 antibody and lysates from transfected cells in A. Immunoblotting was performed with anti-Sik antibody.

**Figure 5. Wildtype Sik and Sik Y-F phosphorylate nuclear proteins that colocalize with GFP Sam68 within the nucleus.** NMuMG cells were transfected with GFP-Sam68

and wild type Sik (A-D), active Sik (E-H), dominant negative Sik (I-L), or GFP vector and pcDNA3 (M-P). Cells were fixed 24 hours after transfection and tyrosine phosphorylated proteins were localized using anti-phosphotyrosine antibodies (B, F, J, N). DAPI was used to stain the nuclei (D, H, L, P). In NMuMG cells, Sam68 displays diffuse, nuclear localization visible by green fluorescence (A, E, I). Cells co-transfected with GFP-Sam68 and wild type Sik or Sik Y-F also stain strongly with the anti-phosphotyrosine antibody visualized using rhodamine (B, F), while no phosphotyrosine is detected in cells expressing kinase defective Sik K-M (J). Panels C, G, K, and O are composites of all colors. Co-localization of anti-phosphotyrosine and Sam68 appears as yellow. GFP alone is expressed in throughout the cell (M) and is negative for anti-phosphotyrosine staining (N).

**Figure 6. The Sik SH2 and SH3 domains bind Sam68.**

NMuMG cells were transfected with GFP-Sam68 and pcDNA3 (Vector), wildtype Sik, activated Sik Y-F, and kinase defective Sik K-M expression constructs. Cell lysates were divided equally and incubated with GST, GST Sik SH2/SH3, GST-Sik SH2, and GST-SH3 covalently coupled to beads. Bound proteins as well as an aliquot of total cell lysate from GFP-Sam68 transfected cells was separated by SDS-PAGE and immunoblotted with Sam68 AD1 polyclonal antibody. The migration of GFP-Sam68 and Sam68 are indicated with arrows. GFP-Sam68 and endogenous Sam68 protein in all of the cell lysates bound strongly to the GST-Sik SH2 + SH3 and GST-Sik SH3 fusion proteins. GFP-Sam68 binding to the GST-Sik SH2 domain was detected only in cells transfected with wildtype Sik or Sik Y-F suggesting that phosphorylation by Sik is required for Sik SH2 binding.

**Figure 7. Generation of Sik transgenic mice.**

Top: The construct prepared for microinjection uses 2.1 kB of sequence from the keratin 14 promoter region to drive transcription of a full-length Sik cDNA, including the coding sequence and B1 repeat element. This is followed by the human growth hormone gene, which is not translated but which is used to incorporate normal intron processing and a polyadenylation site. The sequence diagrammed existed as an insert into the pGem 3Z bacterial plasmid vector, but was excised and purified prior to microinjection into blastocysts. Bottom: Southern blot analysis of BamHI-digested DNA probed with labeled DNA randomly primed from the 562-bp SstI fragment of Sik. The top arrow indicates the longer fragment present from endogenous copies of Sik on two chromosomes, while the bottom arrow indicates the 2.1 kB fragment released from the transgene cassette. Estimated copy numbers: #7, 1; #19, 2; #20, 20; #22,1; #45, 2; #50, 1, #60, 4, #64, 1, #70, 12; #77, 13; #84, 2.

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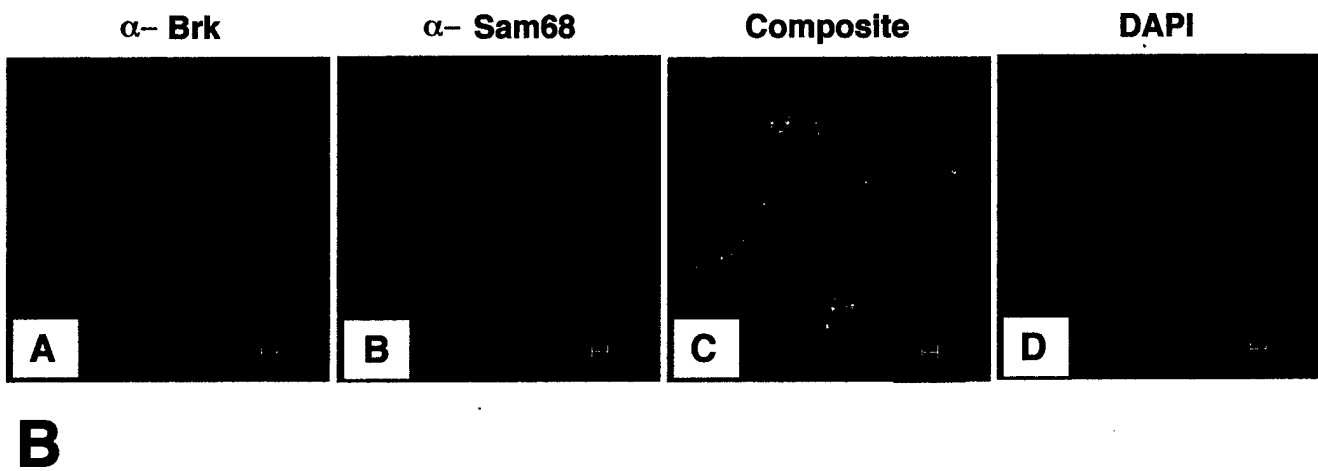
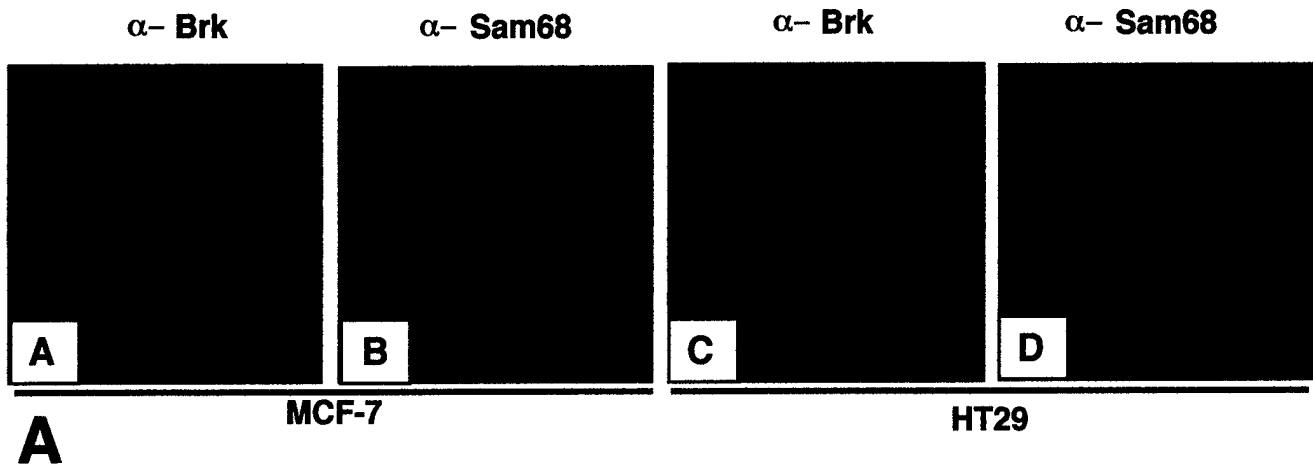
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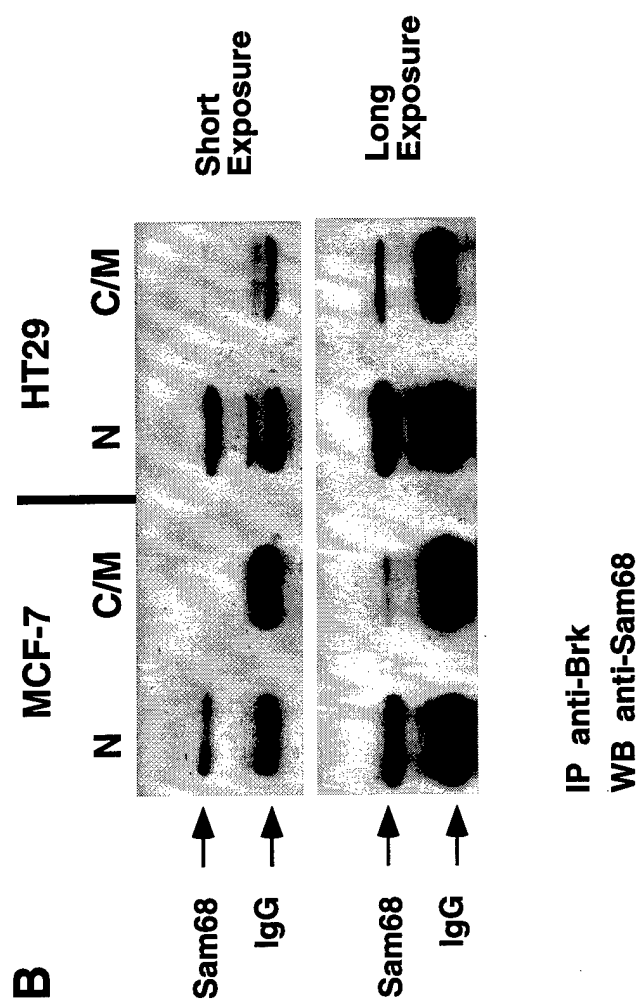
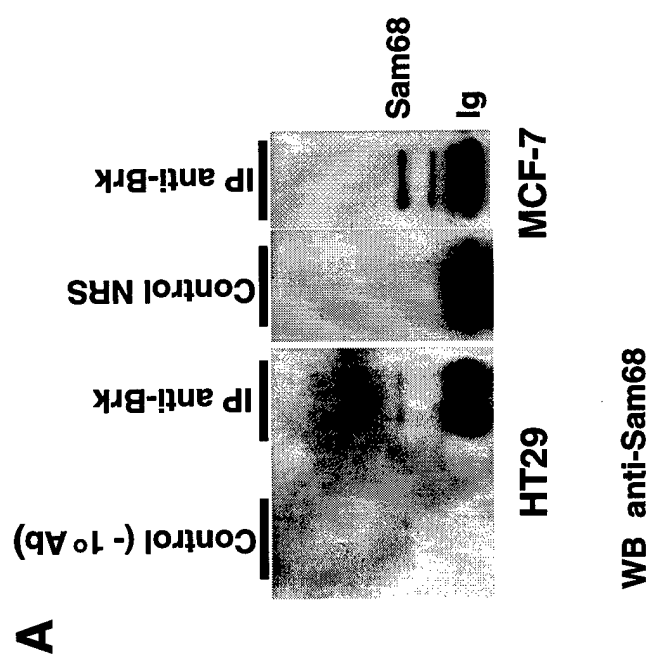
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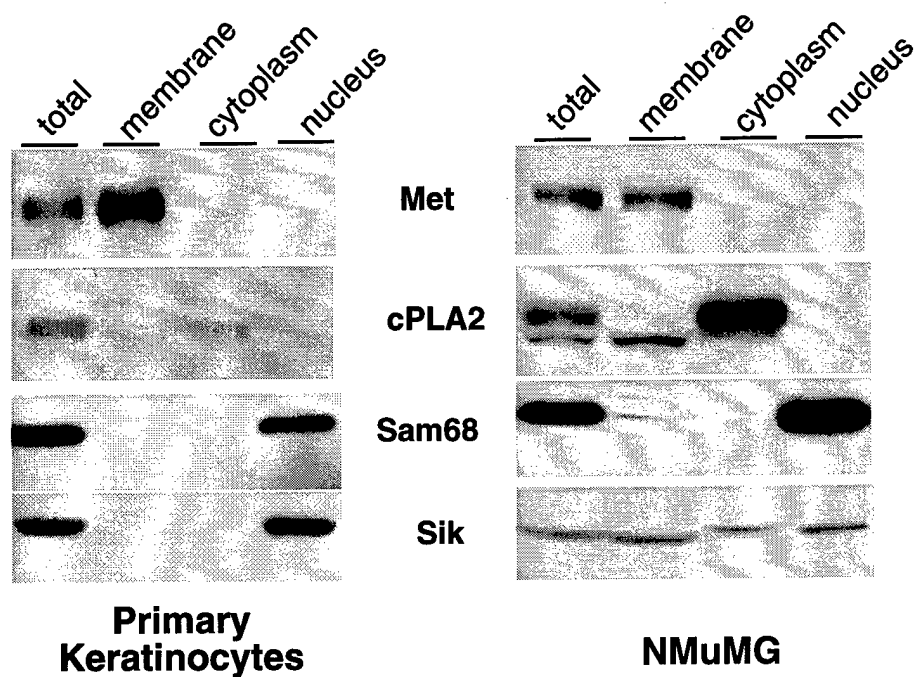
**Figure 1**



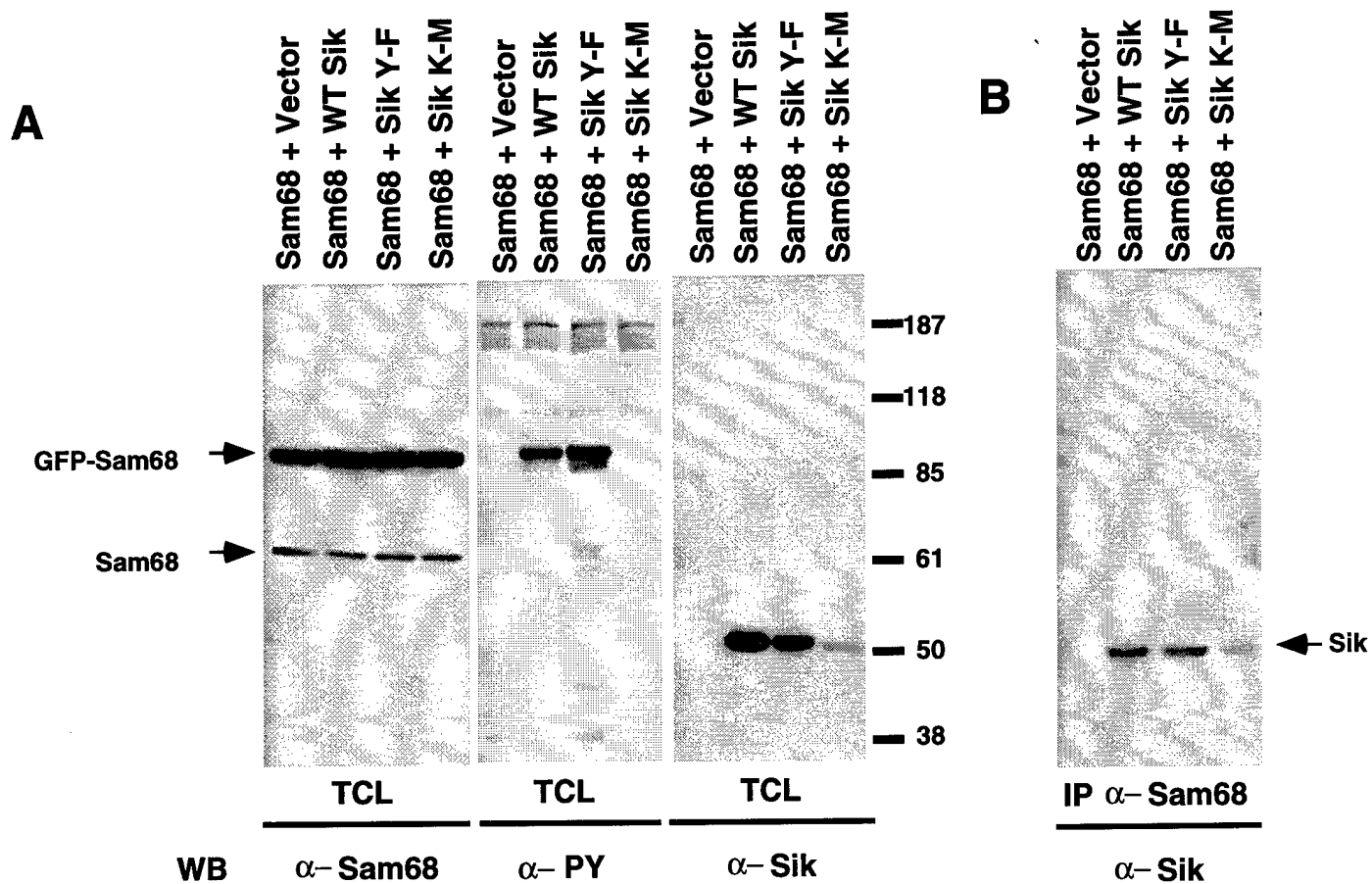


**Figure 2**

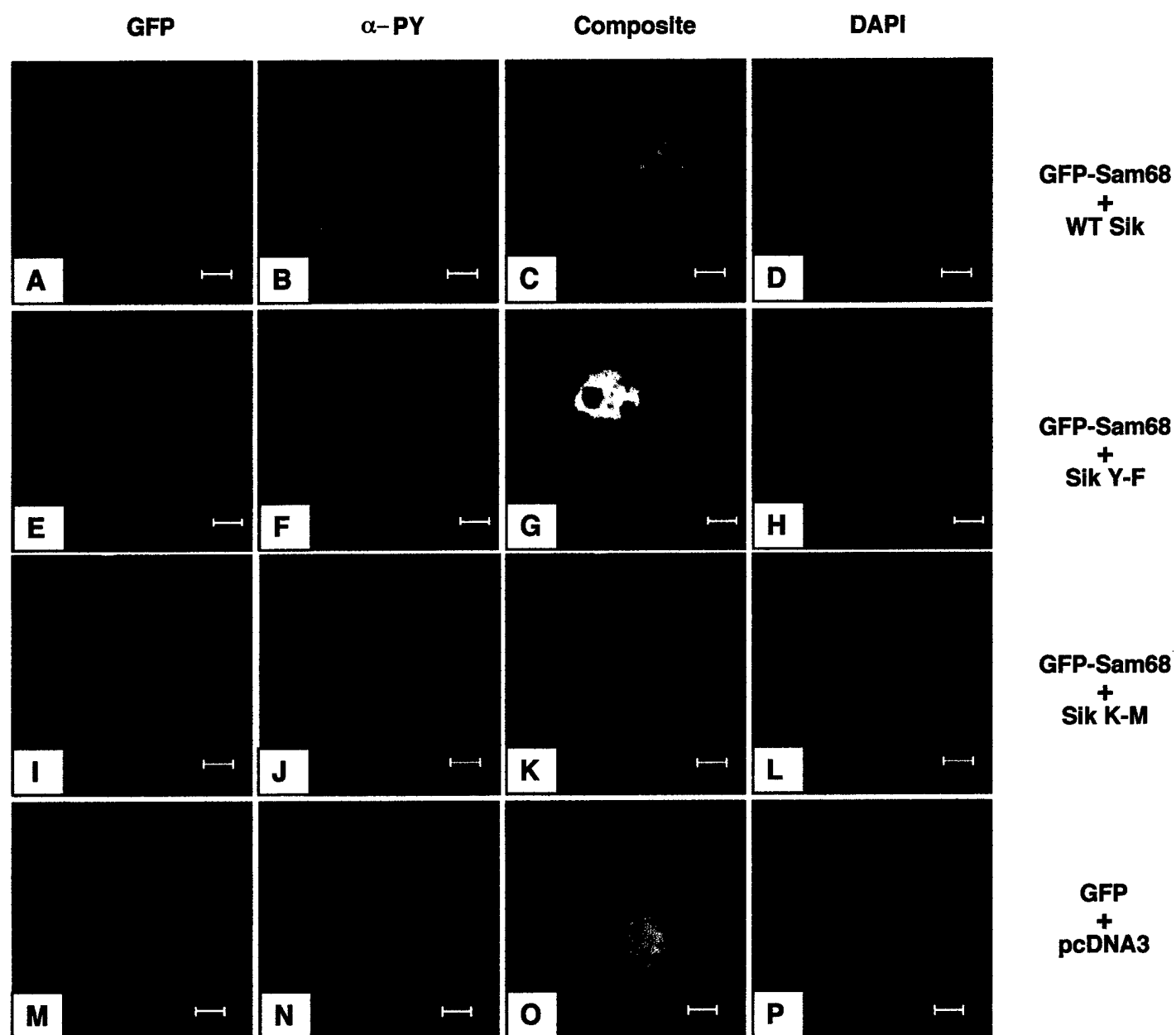
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

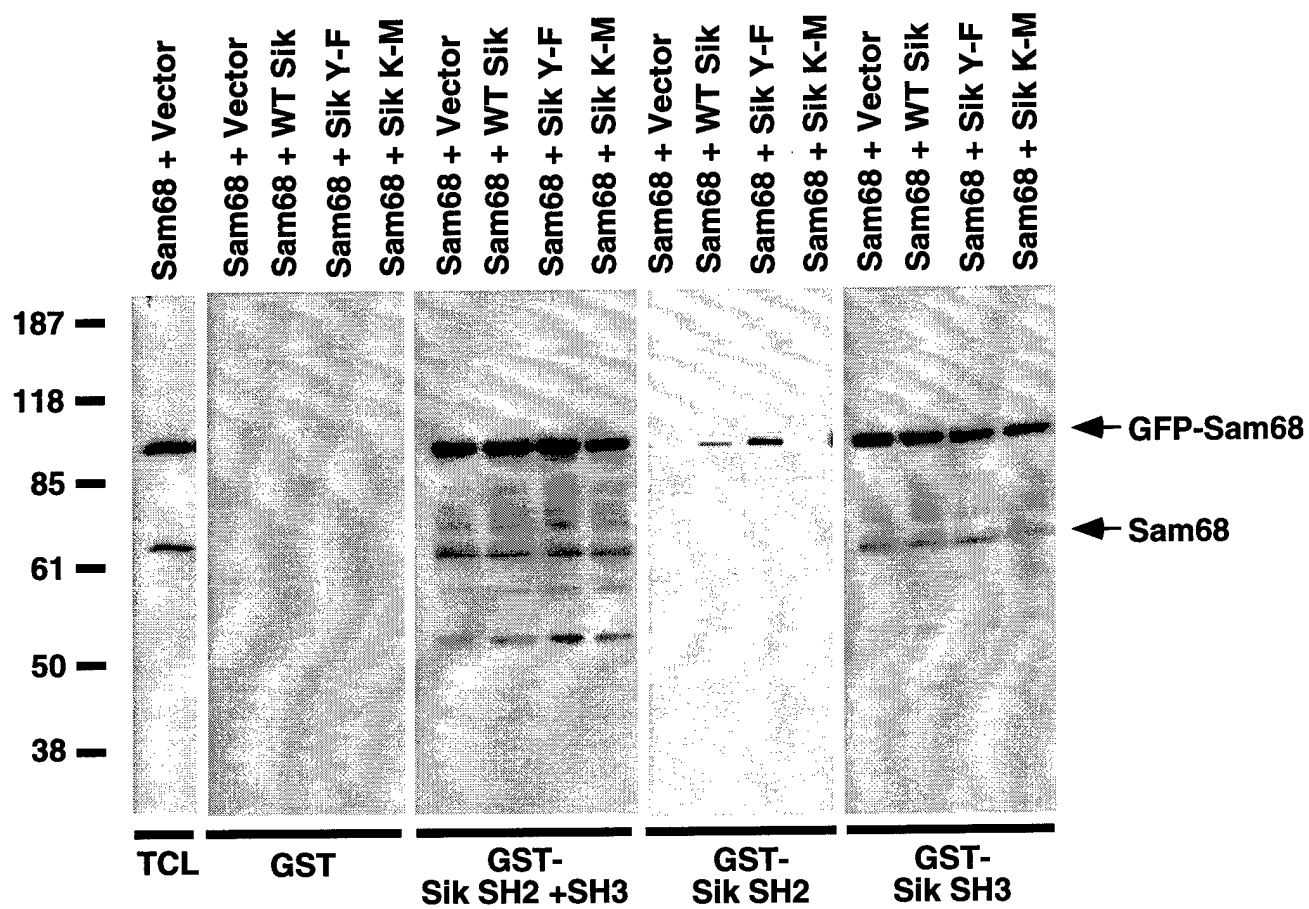


Figure 7

